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Influence of Colipase on the Turbidimetric Determination of Pancreatic Lipase Catalytic Activity[1])

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Summary: The influence of colipase on the turbidimetric measurement of the catalytic activity of pure human pancreatic lipase (EC 3.1.1.3) and of sera from pancreatitis patients was studied. A deoxycholate-stabilized triolein emulsion served as substrate. It was found that the activity of the pure, colipase-free lipase is strongly inhibited by deoxycholate, and can be blocked completely if normal serum, pure human albumin, or the globulin fraction of normal serum is present. The inhibition by serum is competitive. This finding largely excludes the existence of a specific lipase inhibitor in human serum and explains the non-linear response of activity to the amount of serum added, a frequently observed problem with various turbidimetric lipase methods. A high molar excess of colipase (>250-fold) completely abolishes the inhibition of lipase, irrespective of the inhibitory factor studied.

Sera of pancreatitis patients, when measured turbidimetrically without addition of colipase, exhibit elevated lipase activity only if they contain colipase. However, the activity measured is not a function of the serum lipase concentration alone but of the molar ratio of colipase to lipase. Since this ratio varies considerably and is usually too low to ensure complete activation of lipase, erroneously low or even false negative results are obtained. For this reason it is strongly recommended that an excess of colipase is used in turbidimetric lipase assays. It therefore also appears important to study the influence of the serum colipase level on non-turbidimetric lipase methods.

Einfluß von Colipase auf die turbidimetrische Aktivitätsbestimmung von Pankreaslipase

Zusammenfassung: Der Einfluß von Colipase auf die turbidimetrische Aktivitätsbestimmung von gereinigter humaner Pankreaslipase (EC 3.1.1.3) und von Seren von Pankreatitispatienten wurde untersucht. Als Substrat diente eine mit Natriumdesoxycholat stabilisierte Trioleinemulsion. In Abwesenheit von Colipase wird gereinigte Lipase durch die Gallensäure stark gehemmt. Durch Zusatz von Normalserum, gereinigtem Humanalbumin oder der Globulinfraktion läßt sich die Hemmung bis zur Vollständigkeit verstärken. Es ließ sich ferner zeigen, daß die Hemmung durch Serum kompetitiver Natur ist. Dieser Befund schließt das Vorkommen spezifischer Lipaseinhibitoren im Serum praktisch aus und erklärt zugleich das Phänomen der nichtlinearen Beziehung zwischen eingesetzter Probenmenge und gemessener Aktivität bei turbidimetrischen Lipasebestimmungen. Hoher molarer Überschuß an Colipase (>250-fach) hebt die Inhibierung des Enzyms vollständig auf.

Seren von Pankreatitispatienten ergeben daher in turbidimetrischen Verfahren ohne Colipasezusatz nur dann erhöhte Aktivitäten, wenn sie selbst Colipase enthalten. Für die gemessene Lipaseaktivität ist somit nicht allein die Lipasekonzentration, sondern das molare Verhältnis von Colipase zu Lipase im Serum ausschlaggebend. Da dieses Verhältnis stark variiert und für eine vollständige Aktivierung des Enzyms in der

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Regel zu niedrig ist, werden falsch niedrige oder sogar falsch negative Aktivitäten gemessen. Aus diesem Grunde muß dringend empfohlen werden, Lipaseaktivitätsbestimmungen mittels turbidimetrischer Tests nur in Gegenwart eines Überschusses von Colipase durchzuführen. Analog dazu erscheint es wichtig, auch bei nicht-turbidimetrischen Verfahren den Einfluß des Serumcolipasegehalts zu prüfen.

Introduction

Among the numerous methods proposed for assaying serum lipase, turbidimetry has found wide application in the clinical laboratory (1–4). A turbidimetric lipase test is also used on the automatic clinical analyzer® (aca) of Du Pont, which was evaluated by *Forsmann* et al. (5). The radial enzyme diffusion method described by *Goldberg & Pagast* (6) also belongs to this type of assay. However, all these turbidimetric methods reveal a number of problems, e.g. the non-linearity of the clearing reaction with time or the non-linear response to the amount of serum added. The latter observation has repeatedly been ascribed to the existence of lipase inhibitors in serum (5–8).

Until now the evaluation of turbidimetric lipase assays has only been carried out with crude pancreatic extracts, duodenal juice or serum from pancreatitits patients as enzyme sources, but never with purified human lipase, which would allow an exact evaluation of the reliability of these methods. Such a study is of particular importance with regard to the present state of knowledge of the catalytic properties of this enzyme and its complex interactions with its natural substrate, with bile acids and with proteins.

Bile acids as well as proteins have repeatedly been reported to be strong inhibitors of pancreatic lipase (9–13), the mechanism of inhibition being a competition of these amphiphilic compounds with lipase for the access to the lipid-water interface (11, 12). The contradiction between these experimental findings and the fact that lipase physiologically acts in an environment containing high concentrations of bile acids and proteins, was explained during the last decade by the detection of colipase. In the presence of this cofactor, lipase inhibition by amphiphiles is prevented, the role of colipase being that of an anchor for the binding to the lipid water interface (11).

In a recent study (14) we demonstrated that colipase is, like lipase, liberated into the circulation in acute pancreatitis. However, the molar ratio of colipase to lipase was found to be in the range from <0.034 to 2.14 in these sera. Since lipase activity, when measured in assays with high bile acid concentration, is strongly dependent on the degree of saturation with colipase (11, 14. 15), we concluded that serum lipase activity determined by turbidimetry is mainly a function of the colipase content of a serum sample and not of the true lipase concentration (14).

The aim of the present investigation therefore was:

1. to study the importance of colipase for turbidimetric lipase determination, using pure human lipase in both the presence and absence of serum, and
2. to demonstrate the influence of the serum colipase/lipase molar ratio on the turbidimetric measurement of lipase catalytic activity.

Materials and Methods

Reagents

Sodium deoxycholate was purchased from Sigma-Chemie, Munich, FRG, sodium glycocholate from Calbiochem-Behring-Corp., La Jolla, CA, U.S.A. Triolein (95% pure) was obtained from Serva-Chemie, Heidelberg, FRG, and used without further purification. Human serum albumin was from Travenol, Munich, FRG. Gum acacia was obtained from Merck, Darmstadt, FRG. Human pancreatic lipase was purified to electrophoretic homogeneity from pancreatic tissue. Pure colipase was isolated from pig pancreatin (grade VI, Sigma-Chemie, Munich, FRG) as described in detail previously (14).

Methods

Enzyme activity determinations were performed at 25 °C throughout. Lipase activity was measured titrimetrically with gum acacia-stabilized triolein as substrate, containing 1.5 mmol/l glycocholate (8) and 5 mg/l purified colipase. The final assay volume was 3 ml; the titrant was 0.01 mol/l NaOH. The equipment (autotitrator TTT 1 c, automatic burette ABU 12, recorder Servograph REC 261) was from Radiometer, Copenhagen, Denmark. 1 U of lipase activity is defined as 1 micromole fatty acid titrated per min. Our purified preparation of human pancreatic lipase had an activity of 2850 U/mg enzyme under these conditions.

For turbidimetric lipase determination a 0.25 mmol/l triolein emulsion in 20 mmol/l sodium deoxycholate, 25 mmol/l Tris-HCl buffer pH 8.6 was used (14). The substrate solutions according to *Vogel & Zieve* (2) and *Shihabi & Bishop* (3) were prepared by exactly following the procedure described by these authors. The aca-triolein emulsion was taken from the plastic bags after a blank run on the aca. Absorbance was monitored at 365 nm using an Eppendorf-photometer with recorder 4412 (Netheler and Hinz, Hamburg, FRG). The final assay volume was 1.040 ml. Serum colipase concentration was determined as described (14). The radial enzyme diffusion assay for lipase was carried out as described by *Goldberg & Pagast* (6).

Protein concentration was determined by a micro biuret procedure (16).

Results

Effect of colipase on the turbidimetric measurement of pure human lipase

Figure 1 shows the clearing of a deoxycholate-stabilized triolein emulsion catalyzed by pure human lipase. With both enzyme concentrations used a non-linear absorbance decrease is obtained, suggesting progressive inhibition of the enzyme. The ratio of ΔA/min for both samples is only about 6.5 during the first minutes and therefore does not correspond to the 100-fold difference in activity. However, if the assay is performed in the presence of a 1100-fold (11-fold respectively) molar excess of colipase over lipase, a striking effect is seen: in the case of the low activity sample, the absorbance decrease is greatly enhanced and becomes practically linear. The effect is even more pronounced with the high activity sample; the clearing reaction becomes extremely fast, impressively demonstrating the dramatic activation of lipase by colipase.

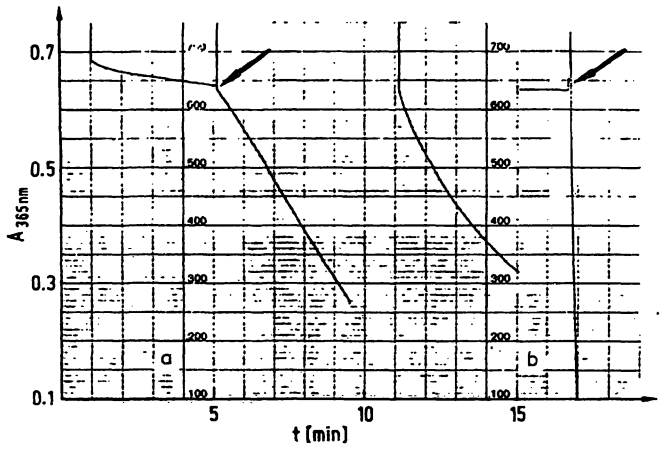

Fig. 1. Activation of pure human lipase by colipase. The reaction was started by the addition of 40 µl enzyme solution containing (a) 35 ng and (b) 3500 ng lipase to 1 ml substrate solution. Arrows indicate the addition of 8 µg pure colipase. Original curves.

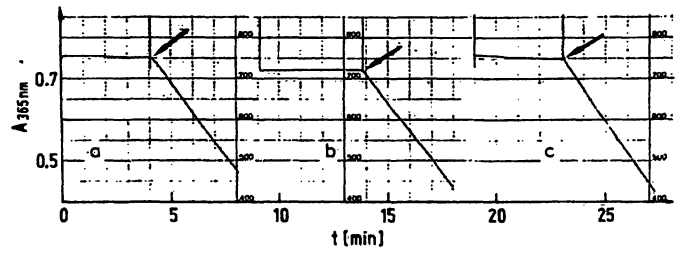

Fig. 2. Inhibition of lipase by albumin (a), the globulin fraction of pool serum (b) and normal serum (c). Final protein concentration 2.7 g/l. The reaction was started with 25 ng lipase. After addition of 8 µg colipase (arrows) activity is restored. Original curves.

Effect of protein on lipase catalytic activity in the absence and presence of colipase

A slow clearing reaction is observed with pure lipase, whereas complete inhibition of the activity was found in the presence of a certain amount of protein (fig. 2a–c). At a final concentration of 2.7 g/l protein, which is usually the concentration in turbidimetric assays if serum is analyzed (0.0385 fraction volume), pure human albumin and a globulin fraction prepared by ammonium sulphate precipitation cause a complete blockage of the enzyme activity. With a pool serum from healthy individuals a slight decrease of absorbance is seen, but it is distinctly lower than that found with pure lipase alone (fig. 1a). However, after addition of colipase to the reaction mixture, lipase activity is immediately restored indicating that the cofactor completely overcomes the inhibitory effect of protein.

Figure 3 shows the effect of normal pool serum on lipase activity in the absence of colipase. It is evi-

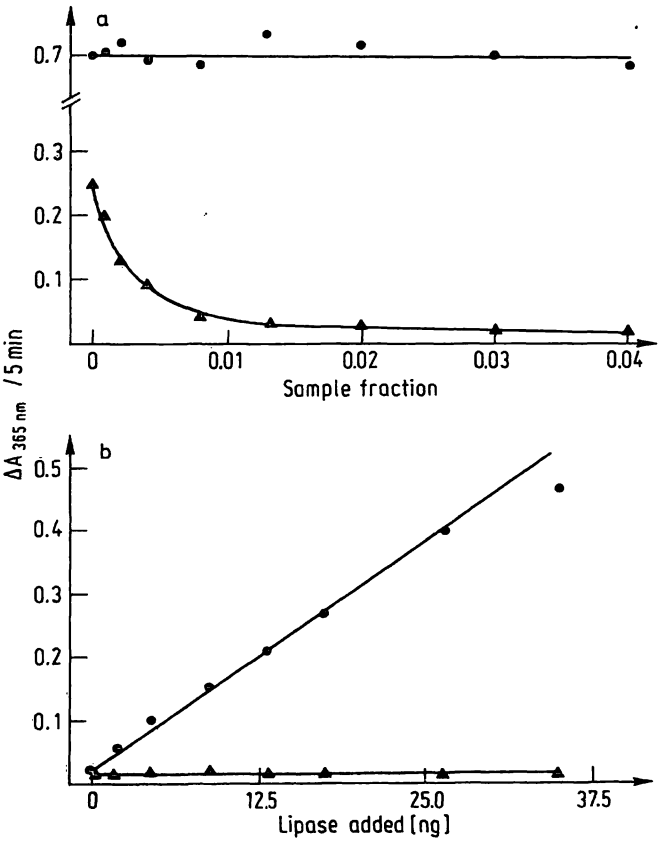

Fig. 3. a) Effect of normal pool serum on lipase catalytic activity in the absence (▲———▲) and presence (○———○) of colipase. Final lipase concentration 50 µg/l, colipase 7.6 mg/l.
b) Effect of increasing amounts of pure lipase added to normal pool serum in the absence (▲———▲) and presence (○———○) of colipase. Fraction volume of serum 0.0385; 7.6 mg/l colipase.

dent, that the extent of the serum effect is dependent on the final serum concentration: lowering the amount of serum while keeping the lipase concentration constant results in a progressive increase in ΔA/min. If the measurement is performed in the presence of colipase, variation of the serum concentration has no influence on the activity (panel a). The strong inhibitory effect of serum is also demonstrated by an experiment shown in panel b of figure 3: if the serum concentration is held constant at 0.0385 volume fraction and increasing amounts of lipase are added, no increase in ΔA/min will be obtained unless an excess of colipase is present.

The inhibition of lipase by serum can be visualized directly by means of the enzyme diffusion method for serum lipase determination as proposed by *Goldberg & Pagast* (6). In this assay agar serves as mechanical support for the substrate which consists of a deoxycholate-stabilized triolein emulsion. Two serum samples containing lipase were placed in wells (fig. 4) and the agar dishes were incubated for 3 h at 37 °C. After that time a zone of turbidity remained around the well while a clearing zone — due to complete hydrolysis of triolein — had developed at a certain distance from the centre, although the lipase concentration is considerably lower in that area than in the region close to the well. This effect was called the "bulls eye phenomenon" (6). However, if the experiment was performed with the same samples with prior addition of colipase, the "bulls eye phenomenon" disappeared and clearing was now seen within the whole diffusion area.

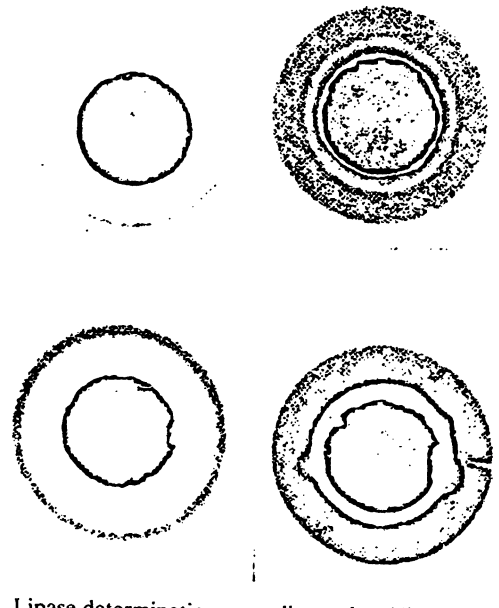

Fig. 4. Lipase determination according to l.c. (6). Left wells with two pancreatitis sera (381 U/l above, 2470 U/l below) showing the "bulls eye phenomenon". Right wells: disappearance of the effect when colipase at a final concentration of 16 mg/l had been added to the sera.

By means of the pH-stat technique we found that the inhibition of lipase by human serum is competitive, as shown by the *Lineweaver—Burk* plot in figure 5. Curve 4 in figure 5 was obtained when the measurements were carried out in the presence of colipase, irrespective of whether pool serum was present or not.

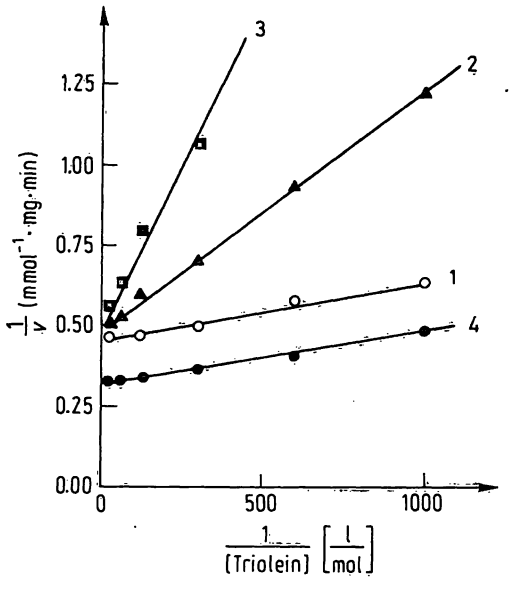

Fig. 5. Competitive inhibition of lipase by human serum.
1) pure lipase,
2) 0.02 serum fraction volume,
3) 0.033 serum fraction volume,
4) conditions as in 1, 2, 3, but with colipase at a final concentration of 5 mg/l.
Emulsified triolein in 20 mmol/l deoxycholate served as the substrate. pH-stat technique.

Dependence of lipase catalytic activity on colipase concentration

Full reactivation of bile acid-inhibited lipase by colipase has been reported for equimolar conditions (17) or with a slight molar excess of colipase over lipase (15). In contrast to these results, which were obtained by continuous titrimetric techniques, we found a 250-fold molar excess of colipase to be necessary for complete reactivation of lipase under the conditions of our turbidimetric assay, as shown in figure 6. At equimolarity only 38%, at twofold excess only 60% of the maximal activity is recovered.

Measurement of lipase catalytic activity in pancreatitis sera

As demonstrated in figure 6, lipase catalytic activity assayed turbidimetrically is highly dependent on the degree of saturation of lipase with colipase. The mo-

lar ratio of colipase to lipase in the sera of 25 patients with acute pancreatitis was found by us to be in the range of < 0.034 to 2.14 (14) indicating non-saturation (with regard to turbidimetry) of the enzyme with its cofactor in any of the sera studied. Consequently lipase catalytic activity, when measured turbidimetrically, has to be found erroneously low in all sera, the deviations from the "true" activity increasing as the serum colipase/lipase ratio decreases. This fact is demonstrated in figure 7. Three pancreatitis sera with different colipase/lipase ratios were analysed with our turbidimetric assay and the influence of an excess of colipase on the course of the clearing reaction was studied. Serum a (lipase catalytic concentration 4510 U/l, molar colipase/lipase ratio 2.14) was stimulated about 1.7-fold, serum b (6352 U/l, ratio 0.46) 3.8-fold and serum c (2811 U/l, ratio 0.04) 13.8-fold by an excess of colipase. It is evident that without saturating amounts of the cofactor no correlation exists between the absorbance change and the lipase catalytic activity as measured titrimetrically. It should be emphasized particularly that with serum c even a false negative result is obtained, as judged from comparison with the ΔA/min determined with normal human serum. The correct relationship between lipase concentration and the photometric signal is obtained only in the presence of an excess of colipase.

To confirm that our findings are of general validity regarding all turbidimetric lipase assays and not only the one we used, we carried out a set of similar experiments using the substrate formulations of *Shihabi & Bishop* (3), *Vogel & Zieve* (2), and, in addition, the substrate emulsion from the aca. These assays contain the same compounds as ours (tris buffer, deoxycholate and triolein), but there are differences in the concentrations. However, as exemplified by figure 8, the same qualitative results were obtained.

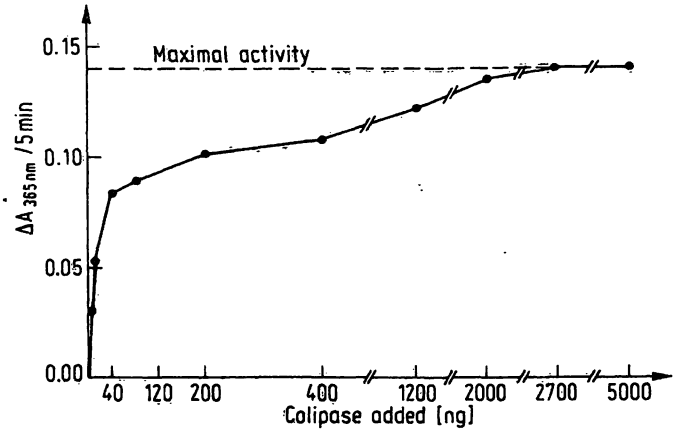

Fig. 6. Activation of lipase by increasing amounts of colipase. After addition of 20 µl colipase solution the reaction was started with 40 µl normal pool serum containing 50 ng pure lipase. Final assay volume 1.06 ml.

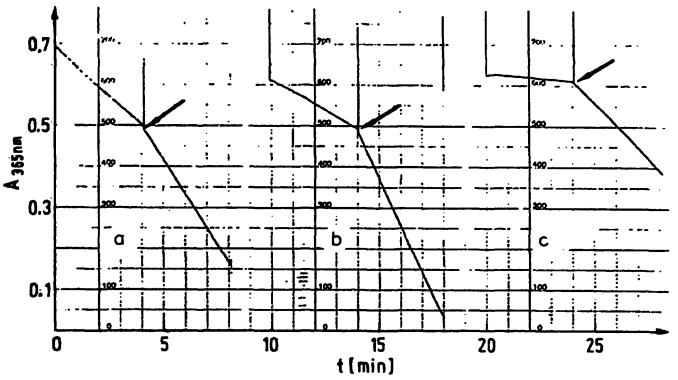

Fig. 7. Activation of three sera from pancreatitis patients with different colipase/lipase ratios (see text) by an excess of colipase (8 µg). Arrows indicate the addition of the cofactor. 20 µl serum was added to 1 ml substrate emulsion. Original curves.

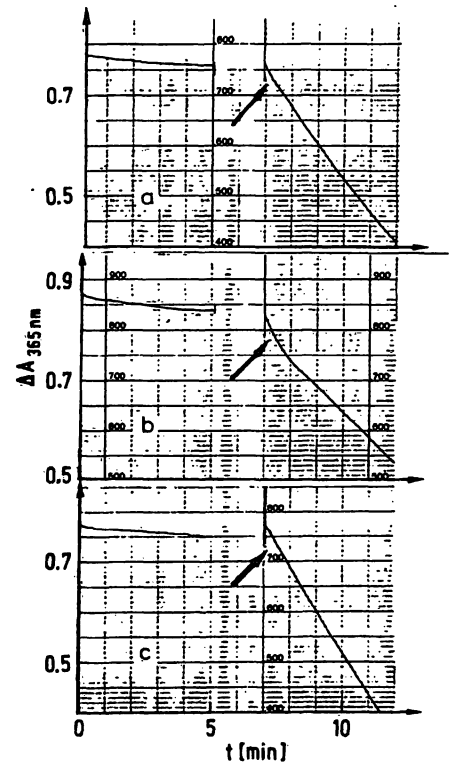

Fig. 8. Comparison of the turbidimetric lipase methods according to
a) *Vogel & Zieve* (2),
b) *Shihabi & Bishop* (3) and
c) the procedure for the aca.
30 µl of serum (2811 U/l) with a colipase/lipase ratio of 0.04 were added to 1 ml of substrate emulsion. Arrows indicate the addition of 8 µg colipase.

Discussion

Our results clearly demonstrate that turbidimetric methods for serum lipase catalytic activity that do not employ an excess of colipase lead to unreliable results, and they should therefore no longer be used. This finding, however, is not surprising in the light of the present understanding of the mechanism of lipase action.

It has been known for a long time that bile acids are strong inhibitors of pancreatic lipase, although at submicellar concentrations they can also function as activators. The extent of inhibition is not determined by the absolute bile acid concentration but by its concentration relative to the substrate concentration or substrate surface area (17): the higher the molar ratio of bile acid to substrate the greater the extent of inhibition. This ratio is especially unfavourable in turbidimetric methods because a low substrate concentration for the photometric monitoring of the clearing reaction is required. Deoxycholate is the bile acid preferred for turbidimetric assays, obviously because it is a less powerful inhibitor than the conjugated tri- and dihydroxy compounds (10, 17).

It is of practical importance that the low activity of lipase towards deoxycholate-stabilized triolein can completely be blocked by whole serum or serum fractions. This effect has been noted previously by several authors (6, 7, 13) and was repeatedly attributed to the presence of specific lipase inhibitors in serum. Four such inhibitors of different molecular weights were fractionated on Sephadex G-200 (7). *Rick* (8) found that the inhibitory capacity of serum is partially dialysable. The main argument of other authors for the existence of serum lipase inhibitors was the observation that upon dilution pancreatitis sera exhibit higher lipase activities than expected from the measurement with the undiluted sample (2) or from non-ideal recoveries (5, 6). This conclusion, however, is not plausible, since the molar relationship of both, lipase and of a lipase inhibitor, would not be altered upon dilution.

On the basis of recent experiments on the inhibition of porcine lipase by some proteins (12, 18) and our finding that serum competitively inhibits human lipase, it appears reasonable to assume that there is a general physical effect of proteins on lipase action rather than a specific inhibition of the enzyme: inhibition by serum is due to competition of proteins with lipase for the substrate-water interface. That is why turbidimetric assays with low substrate concentrations are highly susceptible to variation of the protein content of the sample. Titrimetric methods, in which high substrate concentration is used (e.g. 0.137 mol/l in *Rick*'s method (8)) are less sensitive, although non-linear response to the amount of serum has also been demonstrated if the sample volume exceeded a certain volume fraction, i.e. 0.13 (8). The "bulls eye phenomenon" observed in the radial enzyme diffusion assay (fig. 4) can also be explained by protein-inhibition of lipase: since the enzyme molecule has a higher diffusion coefficient than most serum proteins it "escapes" from its inhibitors, and that is why clearing is only seen at a certain distance from the central well. In that area, separation of the enzyme from the bulk of serum proteins is largely complete.

The common inhibitory effect of bile acids and proteins is completely overcome if lipase is assayed in the presence of colipase. A high molar excess – about 250-fold – of the cofactor is necessary for full activity of the enzyme. This figure is considerably higher than that reported by other investigators (12, 19). They, however, used titrimetric methods. The reason for this discrepancy is not yet known.

The results presented here show that there is only an apparent contradiction between our findings on the total blockage of lipase activity by bile acids and serum, and the reported usefulness of turbidimetric methods (2–6). From the results of our previous study (14) and from those of the present paper it should by now be evident that elevated serum lipase activity can in fact be detected by turbidimetry, but only if both lipase and colipase are simultaneously present in the serum. However, since the catalytic activity is mainly dependent on the molar ratio of colipase to lipase and not on the absolute lipase concentration (14), the values obtained do not correspond to the true lipase activity of the sample.

It is obvious, that the source of the enzyme used for the evaluation of turbidimetric assays is eminently important. Some investigators used pancreatic tissue extracts added to human serum as "pancreatitis sera" (2, 3, 21). However, these samples cannot be compared to "normal" sera of pancreatitis patients because of their high colipase content. The so called "lipase verifyer" used for the aca-lipase method also contains a considerable amount of colipase (*W. Junge*, unpublished observation) and does therefore not exhibit the problems associated with serum samples (5).

The consequence of our study is that turbidimetric lipase determinations must be carried out in the presence of high molar excess of colipase in order to obtain reliable and reproducible results. A corresponding method, in which an approximately 1500-fold colipase excess is used, has been suggested by

Ziegenhorn et al. Whether colipase or the serum colipase/lipase ratio also has an influence on the titrimetric lipase determination according to *Rick* (8) is presently under investigation in our laboratory.

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